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Award Number: DAMD17-99-1-9281

TITLE: Estrogen-Mediated Breast Carcinogenesis: The Role of
Sulfation Pharmacogenetics

PRINCIPAL INVESTIGATOR: Araba Adjei, Ph.D.

CONTRACTING ORGANIZATION: Mayo Foundation
Rochester, Minnesota 55905

REPORT DATE: May 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE May 2001	3. REPORT TYPE AND DATES COVERED Annual (1 May 00 - 30 Apr 01)		
4. TITLE AND SUBTITLE Estrogen-Mediated Breast Carcinogenesis: The Role of Sulfation Pharmacogenetics		5. FUNDING NUMBERS DAMD17-99-1-9281		
6. AUTHOR(S) Araba Adjei, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Mayo Foundation Rochester, Minnesota 55905 Email - adjei.araba@mayo.edu		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES This report contains colored photos				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Distribution authorized to U.S. Government agencies only (proprietary information, May 2001). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) Catecholestrogens (CEs) are activated to form stable and depurinating DNA adducts. Adducts formed from 4-hydroxyestrone (4-OHE1) and 4-hydroxyestradiol (4-OHE2), result in mutations that lead to genotoxicity and therefore breast carcinogenesis. Prevention of the genotoxic effects can be achieved in part through the sulfate-conjugation of the CEs, catalyzed by Sulfotransferase (SULT) enzymes. Many of the human SULTs are genetically polymorphic, thus, inherited differences in activities of these enzymes may contribute to the pathophysiology of breast cancer. We determined the activity of 13 recombinant human SULTs with 4-OHE1, 4-OHE2, 2-hydroxyestrone (2-OHE1), 2-hydroxyestradiol (2-OHE2), estrone (E1) and 17- β estradiol (E2). SULT 1E1 had the highest affinity for them all, with apparent Km values of 0.31 and 0.18 μ M for 4-OHE1 and 4-OHE2. Immunohistochemical studies with SULT1E1 antibody on breast tissue block arrays of non-cancer and tumor samples, detected SULT1E1 enzyme protein. Resequencing of SULT1E1 from DNA of 60 Caucasians and 60 African-Americans identified 3 SNPs which changed encoded amino acids: Asp22Tyr, Ala32Val and Pro253His. The corresponding enzyme activity levels ranged from 26 to 69 and to 315% of the wild type respectively. Thus, variations in sulfation of CEs catalyzed by SULTs, may confer variable risks to the development of breast cancer.				
14. SUBJECT TERMS Breast Cancer, Sulfation, Sulfotransferases, Polymorphic Catecholestrogens, Immunohistochemical, Resequencing			15. NUMBER OF PAGES 38	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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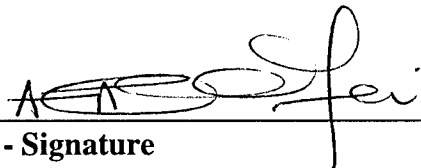
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INTRODUCTION

The catecholestrogens (CEs), 2- hydroxyestrone (2-OHE1), 2-hydroxyestradiol (2-OHE2), 4- hydroxyestrone (4-OHE1) and 4-hydroxyestradiol (4-OHE2) can be metabolically activated to form stable and depurinating DNA adducts. The depurinating DNA adducts are formed from 4-OHE1 and 4-OHE2 and they result in mutations that lead to genotoxicity and therefore breast carcinogenesis. Prevention of the genotoxic effects of these estrogen metabolites can be achieved in part through the sulfate-conjugation of the CEs, catalyzed by Sulfotransferase (SULT) enzymes. Many of the human cytosolic SULTs are genetically polymorphic, thus, inherited differences in the activities of these enzymes would lead to variations in enzyme activities and variations in the inactivation of the CEs. Therefore the variable formation of the genotoxins, ultimately, may contribute to an individual's risk of developing breast cancer.

BODY

At the time of submission of the first year's annual report, Task 2 was incomplete because the immunohistochemical studies with SULT1E1 antisera had shown non-specific background staining which caused some difficulty in classifying the stain as positive or negative. As a result of this ambiguity, a decision was made to purify the antiserum on Protein A Sepharose CL-4B affinity column.

Task 2 was to use immunohistochemical techniques to localize the SULT isoform(s), which show relatively high affinities for the CEs, in normal and neoplastic breast tissues. 6-12 months.

The purified SULT1E1 antibody was used to repeat the immunohistochemical assays. This time, breast tissue block arrays, produced by Dr. Patrick Roche, a co-investigator on this award, in the Experimental Pathology and Immunostaining laboratory, were used for the immunohistochemistry studies. Three sets of block arrays, two of which had breast tumor tissues while the third block had non-cancer breast tissues were used for this study. As observed in Figure 1 (see Appendix I), approximately 93% of the breast tumor tissues expressed SULT1E1 compared to about 57% of the non-cancer tissues. When stratified for estrogen receptor status (ER+ and ER-), results from our cohort of samples indicate that approximately 85% of the tumors are ER+, while only 15% are ER-. From these ER+ tumor samples, 92% of them stained positively for SULT1E1 antibody, compared to a 100% of the ER- tumors. Of the 57% non-cancer tissue samples that expressed SULT1E1(Appendix I), approximately 71% of them were ER+.

Task 3 was to test the hypothesis that there are functionally significant genetic polymorphisms within the specific SULT gene(s) identified and to develop allele-specific restriction digestion assays for these sequences to enable rapid genotyping. 7-24 months.

As mentioned in the previous annual report, the Task 3 project was initiated with the resequencing of SULT1E1 as a means of seeking the common variant alleles of this gene, that are functionally important. At that time, we used a subset of 90 DNA samples, (M90PDR), of the DNA Polymorphism Discovery Resource from the Coriell Institute. This DNA, comprised samples from different ethnic backgrounds that had been stripped of all identifiers. Of great importance in some research of today, is the knowledge of the ethnic background of the DNA in use. For example, valuable information with regards to allele types and frequencies in the different ethnic groups that may have important biological and or medical consequences could be lost. Therefore, in this case, to obtain meaningful data with regards to types of alleles and their frequencies, we repeated the resequencing project, this time with 120 DNA samples. Sixty of these DNA samples are from a subset of a 100 sample PDR from Caucasians and the other 60 from a similar subset from African- Americans. All the eight (8) exons of SULT1E1 have been amplified using M13-tagged intron-based primers located approximately 100 to 150bp upstream and downstream of each exon. The PCR products were sequenced using the dye-primer method--- a sequencing method that identifies unequivocally, the heterozygous nature of amplicons as well as the presence or absence of any insertion/deletion event. The computer program, Polyphred, was used to analyze all the sequences. A total of 806520 bp were analyzed. The results of resequencing are shown in Figure 2 and Table 3. Twenty-three (23) single nucleotide polymorphisms (SNPs) were identified, most of them present in the introns. Five SNPs were detected in the coding region, 3 of which changed encoded amino acids in exons 2 and 7. The Asp22Tyr was found in the African-American DNA, and Ala32Val and Pro253His were detected in Caucasian samples. The allele frequency for each of these SNPs is 0.008%.

Expression constructs were made for each of these 3 mutations for transfection into COS-1 cells by the transfast method. Prior to this transfection, the presence of those mutations was validated by new independent PCR amplifications of the original DNA samples. The resulting recombinant SULT1E1 protein catalyzed reactions with estradiol to varying extents. Estradiol was used for the assays because it is the most commonly used substrate to determine exclusively, the activity of SULT1E1. Moreover, it is the precursor of the CE, (4-OHE2), that has been implicated in carcinogenesis. Figure 3 shows the enzyme activities for these mutants as a percentage of the SULT1E1 wild type activity. The Asp22Tyr variant, found in the African-Americans, gave an activity level that is about 26% of the wild type activity, while the Ala32Val variant, found in Caucasians was 69% of the wild type activity. On the other hand, the Pro253His activity level was about 315% that of the wild type. Based on this genotype –phenotype correlation, we have designed primers to amplify exons 2 and 7 for assays based on restriction sites introduced into the DNA sequence by the variant nucleotides. An Ssp1 restriction site was introduced in exon 2 as a result of the Asp22Tyr change, while an Mwo1 site was removed due to the Ala32Val change. Because the Pro253His change did not introduce any new restriction site in exon 7, a DraIII site has been incorporated in the primers

designed to amplify exon 7. Digestion with DraIII will differentiate the wild type from the variant amplified DNA.

KEY RESEARCH ACCOMPLISHMENTS

1. After affinity purification of the SULT1E1 antiserum, the immunohistochemical studies were repeated and SULT1E1 was expressed in breast tissues from both non- cancer and tumor samples. This completed work for Task 2.
2. We resequenced 120 DNA samples and analyzed a total of 806520 bp of sequence information. We identified 23 SNPs including 3 in the coding region of SULT1E1 that changed encoded amino acids. We performed one type of genotype-phenotype correlation, i.e. comparing the functionally important genotypes with levels of enzyme activity. We are repeating a second phenotype correlate i.e. determining quantity of immunoreactive protein, as an additional important information for Task 3. Finally, we are validating the allele-specific digestion assay using the specified restriction enzymes.

REPORTABLE OUTCOMES

A manuscript entitled CATECHOL ESTROGEN METABOLISM: POSSIBLE ROLE FOR SULFATION AND CARCINOGENESIS was prepared for a short communication to Drug Metabolism and Disposition, however based on new additional data, the manuscript is being reformatted for submission as a full paper to Carcinogenesis. See Appendix II for attached Draft manuscript and the additional results for Task 1.

CONCLUSIONS

As a step towards identifying whether sulfate –conjugation catalyzed by SULTs may represent an independent risk factor for the development of breast cancer, we have determined that the catecholestrogens are substrates for most of the SULTs. We also determined that SULT 1E1 had the highest affinity for 4-OHE2, the CE responsible for forming the genotoxins that form carcinogens. SULT1E1 is expressed in breast tissues from both non-cancer and tumor samples. SULT1E1 is also polymorphic. Two of the 3 variant alleles that we discovered, change encoded amino acids and resulted in variations in levels of enzyme activity.

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Zhang, H., Varmalova, O., Vargas, F.M., Falany, C.N. and Leyh, T. Sulfuryl Transfer: The Catalytic Mechanism of Human Estrogen Sulfotransferase. *J. Biol. Chem.* **273**(18): 10888-92 (1998).

APPENDICES

Appendix I :

FIGURES AND TABLES.

Figures

- Figure 1. Breast tissue block array Immunohistochemical Study: Staining with SULT1E1 Antibody.
- Figure 2. SULT1E1 Polymorphisms: Gene structure and location of single nucleotide polymorphisms (SNPs).
- Figure 3. SULT1E1 Enzyme Activity: Genotype-Phenotype Correlations.

Tables

- Table 1. Apparent Vmax Values for reactivity of some SULTs with 4-hydroxyestradiol(4-OHE2) as substrate.
- Table 2. Apparent Vmax Values for reactivity of some SULTs with estradiol (E2) as substrate.
- Table 3. SULT1E1 Polymorphisms: Frequency of variant alleles in African-Americans and Caucasians.

N.B.

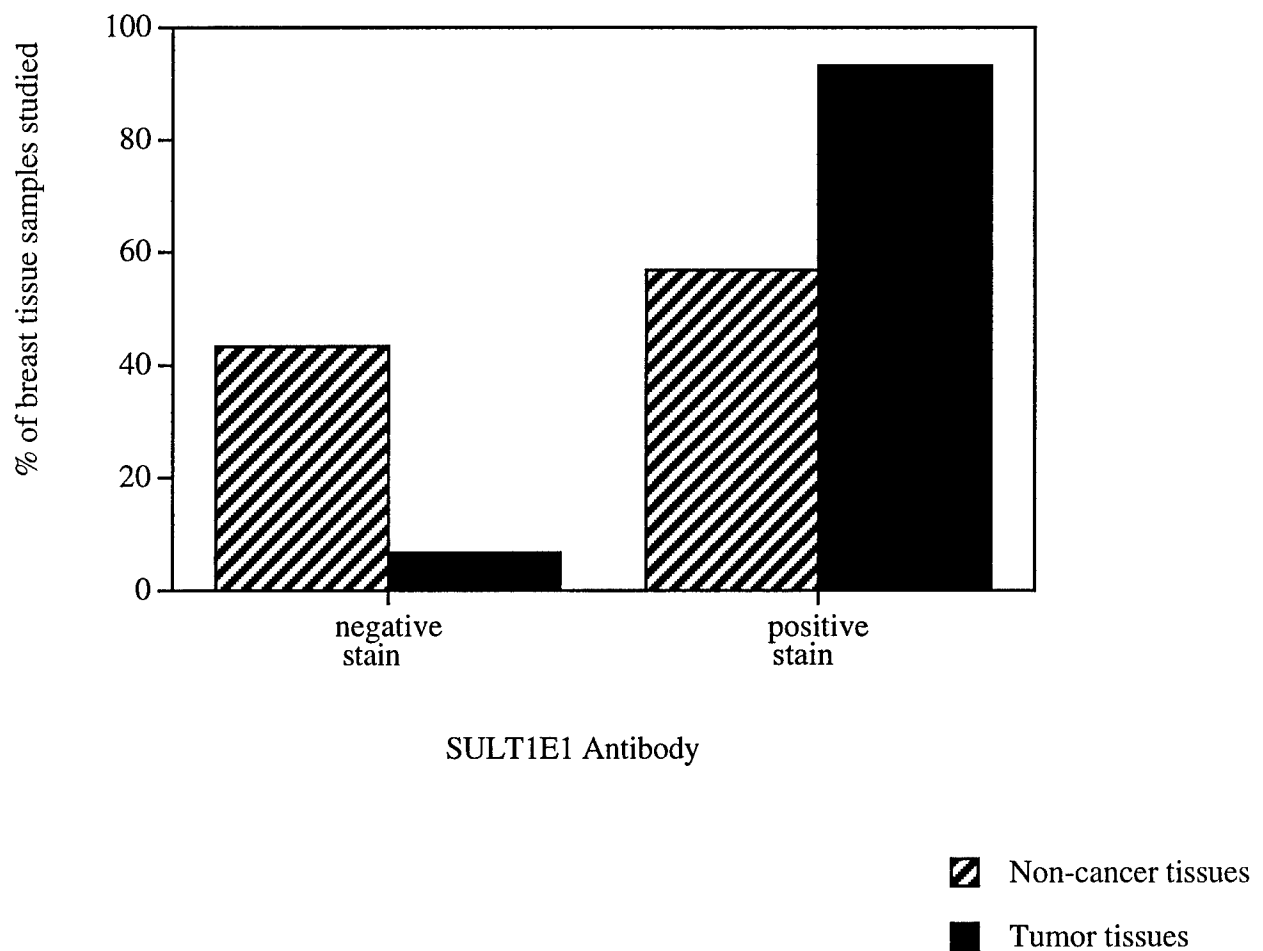
ALL FIGURES AND TABLES IN APPENDIX I PAGES 12-17, AND APPENDIX II (the Draft manuscript) ARE PROPRIETARY DATA.

Appendix II : Attachment

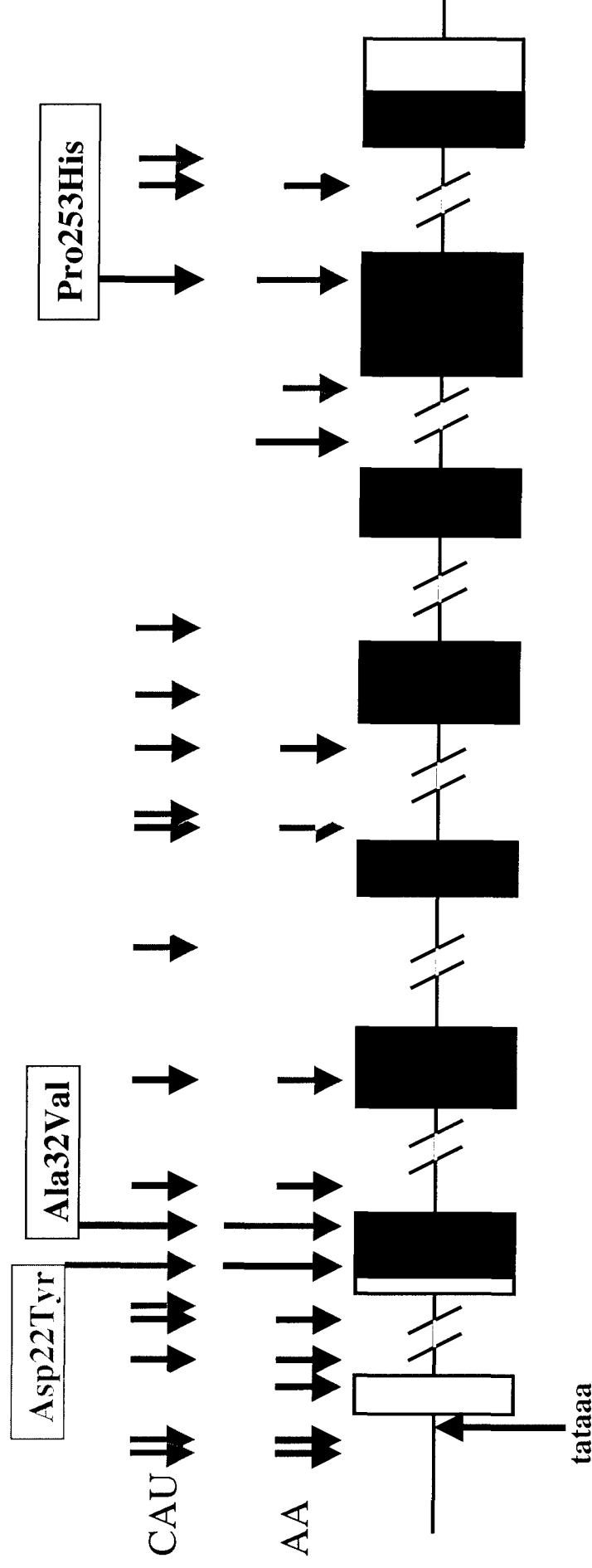
Original Short Communication Manuscript for Drug Metabolism and Disposition.

Manuscript has been changed to a full paper with the addition of more data, and will be submitted to Carcinogenesis.

Breast Tissue Block Array Immunohistochemistry:
Staining with SULT1E1 Antibody.

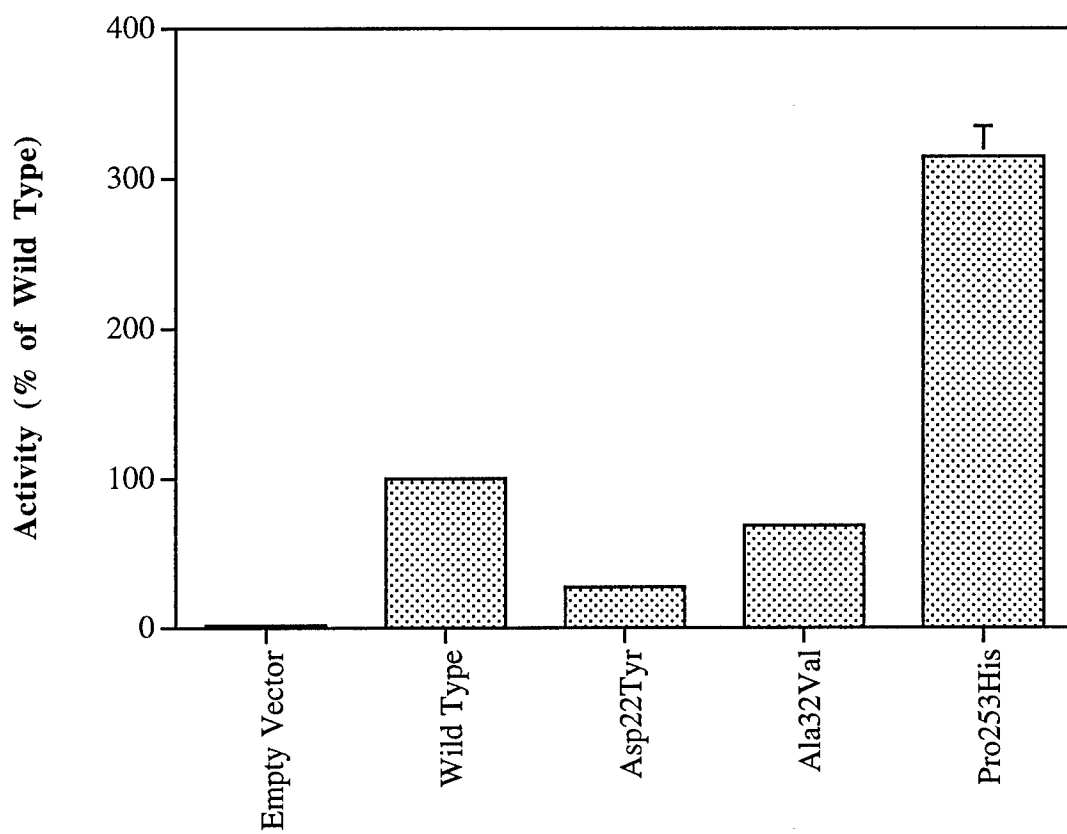


SULT1E1 Polymorphisms



- Allele frequency <1%
- Allele frequency 1-10%
- Allele frequency >10%
- Not seen in this study

**SULT1E1 Genotype-Phenotype Correlation:
Activity Variations due to cSNPs.**



SULT1E1 Constructs

■ SULT1E1 activity

Apparent V_{max} and V/K ratios of SULT isoforms:
Reactions with 4-OHE2.

SULT Isoform	*Apparent V _{max} (nmol/hr/β-Gal)	Apparent K _m (μM)	V _{max} /K _m (x 10 ³)
1A1*1	0.38	44.0	8.6
1A1*2	0.49	23.4	20.9
1A1*3	0.12	6.3	18.5
1A3	0.66	292	2.3
1E1	0.61	0.18	3392
2A1	0.014	41.5	0.3

* Corrected for B-Galactosidase

Apparent V_{max} and V/K ratios of SULT isoforms: Reactions with E2.

SULT Isoform	*Apparent V _{max} (nmol/hr/β-Gal)	Apparent K _m (μM)	V _{max} /K _m (x 10 ³)
1A1*1	0.28	31.3	9.0
1A1*2	0.38	84.6	4.5
1A1*3	0.06	21.1	2.7
1A3	N.D	-	-
1E1	3.52	0.097	36249
2A1	0.093	12.4	7.5

* Corrected for B-Galactosidase

SULT1E1 Polymorphisms

<u>Location</u>	<u>Nucleotide</u>	<u>Sequence Change</u>	<u>Amino Acid Change</u>	Frequency of Variant Allele	
				<u>African-American</u>	<u>Caucasian</u>
5'-Flanking	-232	G → A		0.008	0.000
5'-Flanking	-190	C → G		0.008	0.000
Exon 1	-63	G → A		0.200	0.067
Intron 1	I1(69)	A → G		0.225	0.492
Intron 1	I1(-73)	C → G		0.300	0.383
Intron 1	I1(-20)	A → T		0.092	0.000
Exon 2	64	G → T	Asp22Tyr	0.008	0.000
Exon 2	95	C → T	Ala32Val	0.000	0.008
Intron 2	I2(22)	T → C		0.008	0.008
Exon 3	237	T → C		0.000	0.008
Intron 3	I3(-137)	T → G		0.017	0.000
Intron 3	I3(-80)	A → G		0.017	0.033
Intron 4	I4(69)	A → T		0.000	0.008
Intron 4	I4(139)	A → T		0.017	0.000
Intron 4	I4(-23)	A → G		0.008	0.000
Exon 5	459	C → T		0.017	0.000
Intron 5	I5(55)	C → T		0.017	0.000
Intron 5	I5(-10)	C → G		0.083	0.117
Intron 6	I6(55)	I/D		0.017	0.033
Intron 6	I6(-39)	T → C		0.000	0.017
Exon 7	758	C → A	Pro253His	0.000	0.008
Intron 7	I7(-121)	C → T		0.333	0.108
Intron 7	I7(-63)	T → G		0.017	0.000

**CATECHOL ESTROGEN METABOLISM: POSSIBLE ROLE
FOR SULFATION AND CARCINOGENESIS**

Araba A. Adjei, Thomas C. Wood and Richard M. Weinshilboum

Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Medical
School/Mayo Clinic/Mayo Foundation, Rochester, MN 55905 USA.

Address all correspondence and reprint requests to:

Dr. Richard Weinshilboum
Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Medical
School/Mayo Clinic/Mayo Foundation
Rochester, MN 55905
Telephone: (507) 284-2246; Fax: (507) 284-9111

e-mail: weinshilboum.richard@mayo.edu

ABSTRACT

The carcinogenic effects of estrogen as a result of receptor-mediated mechanisms are well established. However, a growing body of evidence suggests that estrogens may also be direct genotoxins. Specifically, the catecholestrogens (CEs), 2-hydroxyestrone (2-OHE1), 2-hydroxyestradiol (2-OHE2), 4-hydroxyestrone (4-OHE1) and 4-hydroxyestradiol (4-OHE2) are estrogen metabolites that can be metabolically activated to semiquinones and quinones and form stable and depurinating DNA adducts. The depurinating DNA adducts are formed from 4-OHE1 and 4-OHE2 and they result in mutations that lead to genotoxicity and therefore breast carcinogenesis. Prevention of the genotoxic effects of these estrogen metabolites can be achieved in part through the sulfate-conjugation of the CEs, catalyzed by Sulfotransferase (SULT) enzymes. Many of the human cytosolic SULTs are genetically polymorphic, thus, inherited differences in the activities of these enzymes may contribute to the pathophysiology of breast cancer. As such, we determined the activity of 13 recombinant human SULTs with both 4-OHE1 and 4-OHE2, and also with the other CEs, 2-OHE1 and 2-OHE2 and the parent estrogens, estrone (E1) and 17- β estradiol (E2). All but one of the SULTs studied catalyzed these reactions to varying degrees. SULT 1E1 had the highest affinity for the CEs, with apparent K_m values of 0.31 and 0.18 μ M for 4-OHE1 and 4-OHE2 respectively. These results suggest that any individual variation in the sulfate conjugation of CEs catalyzed by SULTs may represent a risk factor for breast cancer.

INTRODUCTION

The contribution of estrogen to the risk of breast cancer has been investigated primarily as receptor-mediated events that increase cell proliferation and promote tumor progression (Nandi et al., 1995; Castles and Fuqua, 1996; Preston-Martin et al., 1992). There is mounting evidence, however, which suggests that estrogens may also be direct genotoxins (Liehr, 1994; Yager and Liehr, 1996; Cavalieri et al., 1997). This genotoxicity is imparted through catecholestrogens (CEs) -- the estrogen metabolites-- which, together with their reactive oxidative products, the catecholesterogen semiquinones (CE-SQs) and quinones (CE-Qs), are critical intermediates in a pathway leading to estrogen-induced carcinogenesis (Fig 1) (Liehr, 1994; Yager and Liehr, 1996; Cavalieri et al., 1997). The CEs, 2-OHE1, 4-OHE1 and 2-OHE2 and 4-OHE2 are hydroxylated products of E1 and E2. The metabolism of CEs to the CE-Qs via cytochrome P450 oxidase/reductase activity, results in intermediates that covalently bind to DNA and form both stable and depurinating adducts (Yager and Liehr, 1996; Bolton and Shen, 1996; Cavalieri et al., 1997). The stable DNA adducts, formed from 2-OHE1(E2) remain intact unless repaired. However, the formation of depurinating adducts from 4-OHE1(E2) metabolites and DNA give rise to mutations leading to genotoxicity (Cavalieri et al., 1997). Evidence for the carcinogenic effect of 4-OHE1(E2) has been demonstrated in animal organs prone to estrogen-induced cancers such as the hamster kidney (Zhu et al., 1994), and in human benign and malignant breast tumors (Liehr et al., 1996; Castagnetta et al., 1992). Therefore, the 4-OHE1(E2) metabolite, in particular may be important in breast carcinogenesis. In order to eliminate this genotoxic effect, E1, E2, the CEs and their CE-Qs can undergo phase II- type inactivation reactions, notably O-methylation, glucuronidation, glutathione conjugation and sulfation. The most carefully studied inactivation pathway to date is methyl conjugation (Yager and Liehr, 1996; Cavalieri et al., 1997), catalyzed by Catechol-O-methyltransferase (COMT). A genetically polymorphic enzyme, COMT detoxifies the

estrogen derivatives by forming methyl conjugates and preventing the formation of the reactive CE-Qs (Martucci et al., 1993). Moreover, studies performed to elucidate the relationship between COMT polymorphism and the risk of breast cancer, demonstrated that the inheritance of low COMT activity genotype is associated with increased incidence of breast cancer (Lavigne et al., 1997, Thompson et al., 1998). The implications from these studies suggest that the inability of an individual to inactivate the CEs may be critical in determining the extent of genotoxicity and therefore breast carcinogenesis. Sulfate conjugation, for the inactivation of the CEs, may be a competitive pathway to methylation. This pathway is important for the biotransformation of steroids, hormones, neurotransmitters, xenobiotics and many other drugs (Weinshilboum and Otterness, 1994). Besides inactivating target molecules to increase polarity and facilitate excretion, sulfation will activate certain drugs and procarcinogens as well (Meisheri et al., 1988; Watabe et al. (1982). Most of the circulating estrogen in the body is sulfated and formed from the catalysis of estrogens by SULTs enzymes (Pasqualini and Klinc, 1985). The sulfated estrogens may represent an endogenous system important in the regulation of biologically steroid hormones in target tissues (Hobkirk, 1993; Coughtrie et al., 1998). SULT enzymes will inactivate E1 and E2 by forming sulfate conjugates thereby diverting them from the both the receptor-mediated and non-receptor mediated genotoxic pathways (Fig 2). It is therefore conceivable that the CEs, derived from the parent estrogens and part of this system are likely substrates for SULTs. So like COMT, SULT enzymes may form conjugates with the CEs and inactivate them. Hence sulfation, like methylation could prevent the formation of the reactive CE-Qs which have been implicated in genotoxicity and which may lead to carcinogenesis (Liehr, 1994; Yager and Liehr, 1996; Cavalieri et al., 1997). Many of the SULTs are genetically polymorphic (Raftogianis et al, 1997; Raftogianis et al, 1999), so that any variation in activity would imply a variation in inactivation of the CEs and therefore a variation in the formation of genotoxins.

We hypothesized that estrogens and the CEs are substrates for SULTs isoforms and that these enzymes may play an important role in the detoxification pathway for the estrogens and CEs. We also hypothesized that genetic polymorphisms present in the SULTs may represent an independent risk factor for the development of breast cancer. We therefore set out to perform substrate kinetic studies with recombinant proteins from eight of the ten known human cytosolic SULTs, as well as the allozymes reported for SULTs 1A1 and 1A2, to determine which of enzymes are capable of sulfating E1, E2 and the CEs, and also to determine which of the enzymes had the highest affinity for 4-OHE2-- the CE implicated in the carcinogenic effect in breast cancer.

MATERIALS AND METHODS

The CEs, 2-OHE1, 2-OHE2, 4 -OHE1 and 4-OHE2 were purchased from Steraloids (Wilton, NH). ³⁵S-labeled adenosine 3' phosphate 5'-phospho-sulfate ([³⁵S]- PAPS) and [2,4,6,7-³H] Estradiol (³H-E2) (72 Ci/mmol) were from New England Nuclear Life Science Products (Boston, MA). E1 and E2 were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of reagent grade quality.

Recombinant Human Sulfotransferase

The cDNAs of the phenol sulfotransferases and their allozymes SULTs 1A1 *1, *2, *3; 1A2 *1, *2, *3; 1A3; SULT 1C1; the estrogen sulfotransferase, SULT 1E1 and the hydroxysteroid sulfotransferases, SULTs 2A1, 2B1a and 2B1b were individually and separately ligated to the eukaryotic expression vector pCR3.1. COS-1 cells were then transfected with these constructs using the DEAE-dextran method. The cells were washed with 5mL phosphate buffered saline, homogenized for 30 sec in 2mL of 5mM potassium phosphate buffer (pH 6.5), before centrifugation

at 100,000g for 1 hour to obtain the high speed supernatants (HSS). Untransfected COS-1 cells were also prepared in a similar fashion to serve as control.

SULT Assays and Substrate Kinetic Experiments

The enzyme activities were measured by the method of Foldes and Meek (1973) as modified by Campbell et al. (1987) and by Hernandez et al. (1992). The substrate acceptors used for the kinetic determinations were 2-OHE1(E2) and 4-OHE1(E2). Experiments were initiated with substrate concentrations ranging from 10^{-3} to 10^{-8} M. A second set of experiments was performed with 8 different concentrations near the K_m of the substrates. Whenever possible, p- nitrophenol (4-NP) at 4, 50, 100 μ M or 3mM, dopamine at 60 μ M, dihydroepiandrosterone (DHEA) at 5 and 100 μ M and E1 at 5uM were used as positive controls for the various SULT enzymes. An additional control involved assays performed with COS-1 cells transfected with vector alone. All assays were performed in the presence of 0.4uM PAPS. Blanks for the assays did not contain any sulfate acceptor.

A modified version of the alkaline-chloroform extraction procedure (Zhang et al., 1998) was used for assays with 3 H-E2. Specifically, the conjugation reaction was stopped by the addition of 650 μ L of 10 mM KOH and 3mL of chloroform. The solution was mixed on the vortex for 30 sec then 500 μ L of the aqueous phase was removed and counted. One unit of enzyme activity represented the formation of 1nmole of sulfate-conjugated product per hour of incubation at 37°C. All assays were performed in triplicate and the values reported are averages of the three determinations. Apparent Michaelis constants (K_m s) were calculated with the method of Wilkinson (1961) using a computer program written by Cleland (1963).

RESULTS AND DISCUSSION

In order to test our hypothesis that SULTs may detoxify the CEs that react with DNA to produce genotoxins, by forming sulfate conjugates, a total of twelve recombinant SULT proteins were used for the substrate kinetic studies of the estrogens and the CEs. These substrates included E1, E2, 2-OHE1, 2-OHE2, 4-OHE1 and 4-OHE2. The assays were performed under optimal conditions for each SULT isoform using a modification of the method of Foldes and Meek (1973). Because sulfotransferases are subject to profound substrate inhibition, two sets of experiments were performed with each estrogen substrate studied. The initial set of experiments was to narrow down the workable concentration range for the substrates i.e. in the vicinity of maximum activity. This involved using a 10- fold serially diluted solution of these estrogens ranging from 10^{-3} to 10^{-8} M. In the second set of experiments, eight different concentrations of a 2-fold serially diluted solution of the substrates in the range approaching maximum activity, observed during the initial experiments were used to determine the K_m s. A typical substrate curve and a double inverse plot for one of the substrates, 2-OHE2, is shown in Fig 3. Due to the differential precipitation of substrates which is associated with the Foldes and Meek method of assay for SULT enzymes, and also because the recombinant proteins used for these studies were produced at different times (making it difficult to calculate the transfection efficiency for comparison), V_{max} was not determined for most of the enzymes in these studies. However, apparent K_m values were obtained for most of the SULTs tested in these experiments with some notable exceptions (Tables 1, 2 and 3).

The most striking of these SULTs with the highest affinity for any of the estrogens was 1E1. SULT 1E1 catalyzed reactions with 2-OHE1 and 2-OHE2 with apparent K_m values of 0.27 and 0.22 μ M respectively (Table 1). Similarly, it catalyzed the reactions with 4-OHE1 and 4-OHE2 with apparent K_m values of 0.31 and 0.18 μ M respectively (Table 2). The K_m Values for the parent estrogens, E1 and E2 were 0.11 and 0.097 μ M, respectively (Table 3). These results imply that the

detoxification or inactivation of the estrogens and CEs would proceed to a greater extent with SULT 1E1 than with any of the other SULTs as long as this SULT isoform is expressed in the tissue of interest. Moreover SULT 1E1 has a better affinity for the CE, 4-OHE2, which is implicated in the formation of the depurinating adducts, than for the 2-OH derivatives which form stable adducts with DNA (Figs. 1 and 2).

The apparent K_m values obtained when 4-OHE2 was used as substrate varied from 0.18 μM for 1E1 to about 476 μM for 1A3 (Table 2). SULT 1A1, which is ubiquitously expressed sulfated the CEs, so did 1A2 (Tables 1 and 2) but their K_m s were higher than that for 1E1. This suggests that for any SULT isoform to play an important role in sulfating these estrogens in a tissue of interest, it would have to be overexpressed to offset any effect from 1E1, or, SULT 1E1 will have to be absent. SULT 1C1, which belongs to the phenol sulfotransferase family showed little or no activity towards the E1 and E2 and the CEs (Tables 1, 2 and 3). The assay conditions were the same used for the other phenol SULTs, 1A1 and 1A2. While 1A1 and 1A2 require 4 μM of the prototypic substrate, 4-NP, for maximum activity, 1C1 requires 3mM of 4-NP to show activity (Yoshinari et al., 1998). The absence of sulfation of estrogen-like chemicals by SULT 1C1 has been reported recently (Suiko et al., 2000).

The CEs were also substrates for the hydroxysteroid SULTs, 2A1, 2B1a and 2B1b. Their K_m s using 4-OHE2 ranged from 17.5 to 45.2 μM (Table 2). However the enzyme activities (in terms of the net cpm for the substrates, not shown) were low, such that the signal to noise ratios for those activities were below 1.0. These same SULTs, on the other hand showed higher activities with their prototypic substrate, DHEA (Hernandez et al. 1992; Her et al, 1998). The enzyme activity levels (net cpm) (data not shown) obtained using E1 and E2 with the SULT isoforms were significantly less than the activity levels for their prototypic substrates or even the CEs, except for 1E1. The K_m s are shown in Table 2. When ^3H -E2 was used for K_m determination to compare the radioactive

versus the barium precipitation assays, a value of 29 nM was obtained which is lower than 97 nM obtained by the precipitation method—an indication that the precipitation assay may be underestimating K_m values.

Finally, since SULT 1E1 had the highest affinity for these estrogens, one preparation of recombinant SULT 1E1 was used to perform another experiment with E1, E2, 2-OHE1, 2-OHE2, 4-OHE1 and 4-OHE2. Assuming equivalent precipitation using the Foldes and Meek assay, V/K_m ratios were determined (Table 4). The results indicated that E2 is a better substrate for 1E1 than E1. Amongst the CEs, 4-OHE2 is the better substrate for SULT 1E1 followed by 2-OHE2.

On the other hand, 2-OHE1 and 4-OHE1 are poorer substrates. These results suggest that the keto group at the 17 position of the parent E1 and its metabolites may be decreasing the V/K_m ratio.

In summary, as a step towards identifying whether sulfate conjugation catalyzed by SULT enzymes may represent an independent risk factor towards the development of breast cancer, we determined that 2-OH CEs and the 4-OH CEs, are substrates for most of the SULT isoforms. We also determined that SULT 1E1 sulfated the substrates with relatively low K_m values and that SULT 1E1 had the highest affinity for the CE, 4-OHE2-- the CE implicated in the formation of genotoxins that lead to carcinogenesis.

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FIGURE LEGENDS

Figure 1. Role of estrogen in carcinogenesis. Pathways showing receptor-mediated events and catecholesterogen derived non-receptor mediated genotoxicity.

Figure 2. Estrogen- Induced Carcinogenesis: Possible role for Sulfation .

Figure 3. SULT Substrate Kinetics. (A) Plot of v (activity) versus $[2\text{-OHE2}]$. (B) Double inverse plot of $1/v$ versus $1/[2\text{-OHE2}]$. Various concentrations of 2-OHE2 were used in the experiment.

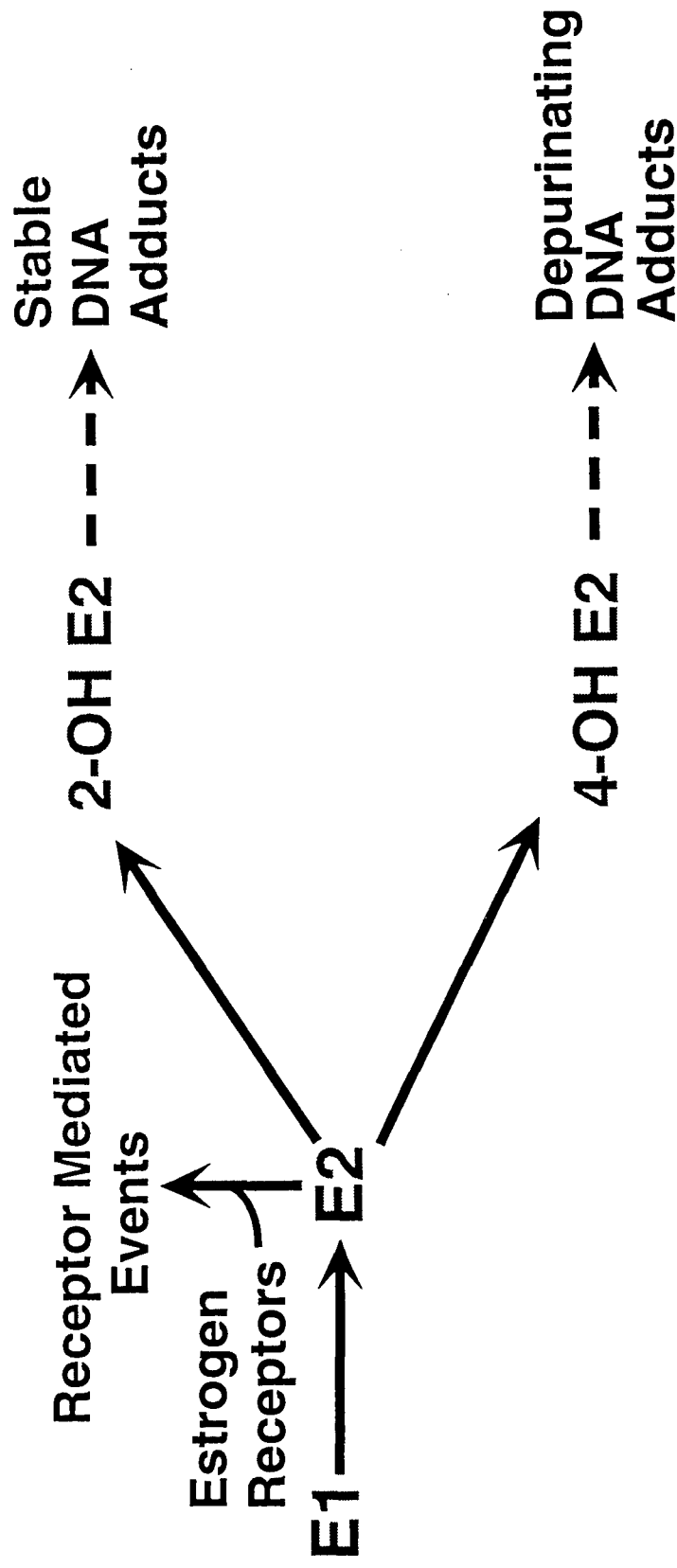
Table 1. Substrate Kinetic Studies. Apparent K_m values obtained for reactions of SULT isoforms and allozymes with 2-OHE1 and 2-OHE2. Values are means \pm S.E.

Table 2. Substrate Kinetic Studies. Apparent K_m values obtained for reactions of SULT isoforms and allozymes with 4-OHE1 and 4-OHE2. Values are means \pm S.E.

Table 3. Substrate Kinetic Studies. Apparent K_m values obtained for reactions of SULT isoforms and allozymes with E1 and E2. Values are means \pm S.E.

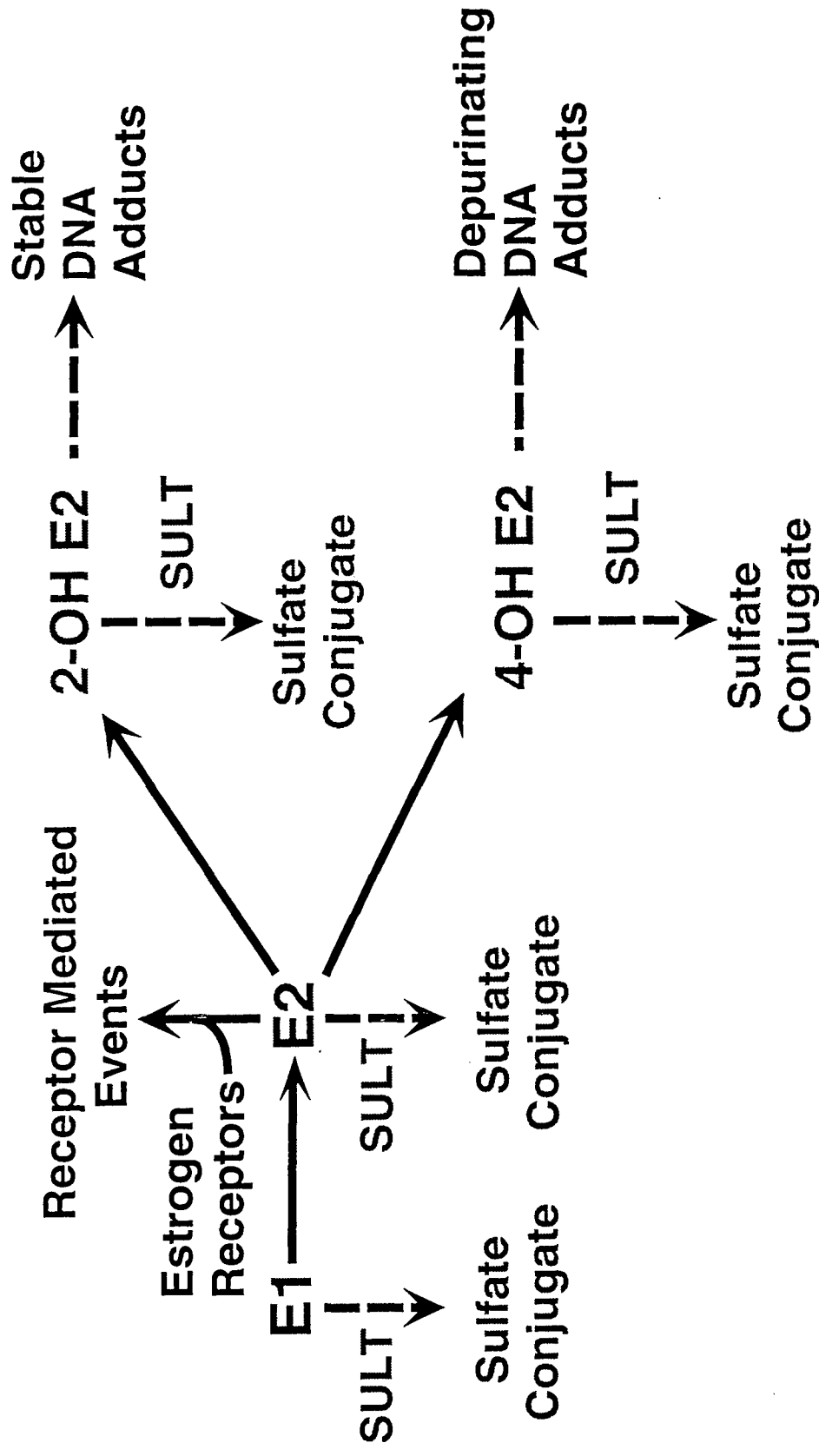
Table 4. Substrate Kinetic Studies. Apparent K_m and V_{max} values obtained for reactions of SULT 1E1 with E1, E2, 2-OHE1, 2-OHE2, 4-OHE1 and 4-OHE2. Values are means \pm S.E.

Estrogen-Induced Carcinogenesis

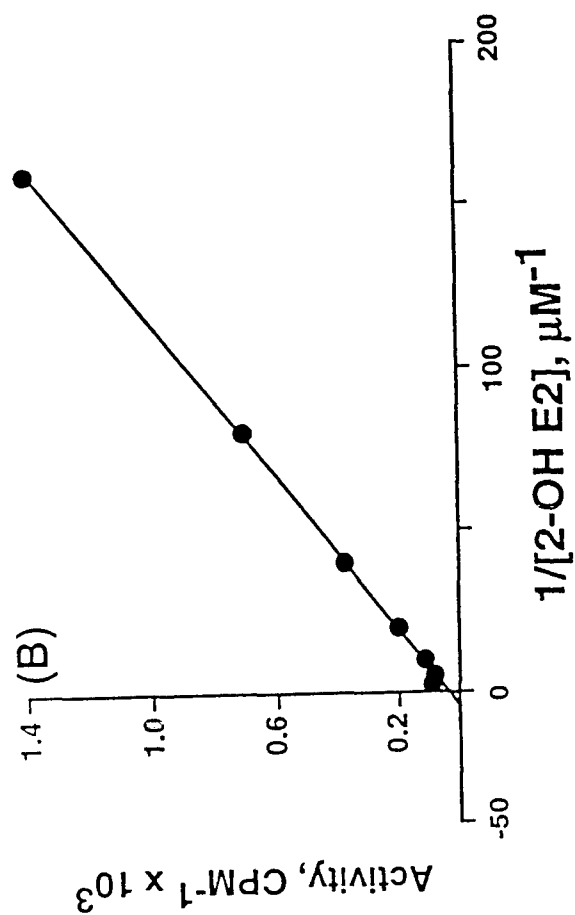
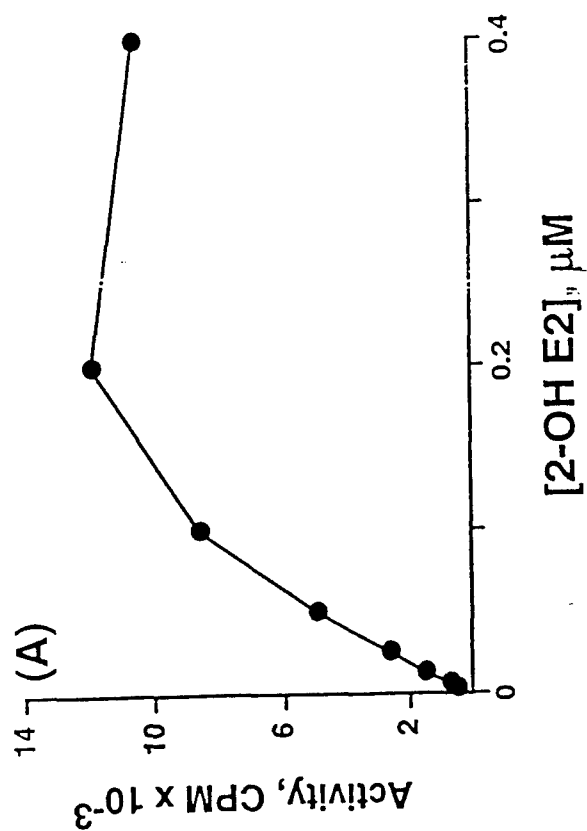


Estrogen-Induced Carcinogenesis

Possible Role for Sulfation



Human SUL1E1 Substrate Kinetics



Estrogen-Induced Carcinogenesis

K_m Values for 2-OH E1 (E2)

SULT Isoform	Apparent K_m Values ($\mu\text{M} \pm \text{S.E.}$)	
	2-OH E1	2-OH E2
1A1*1	5.3 ± 1.3	2.5 ± 0.2
1A1*2	3.6 ± 2.8	11.6 ± 0.5
1A1*3	3.6 ± 0.4	17.3 ± 2.0
1A2*1	19.6 ± 2.4	10.7 ± 1.6
1A2*2	31.5 ± 3.2	484.5 ± 75.1
1A2*3	8.9 ± 1.2	40.8 ± 2.6
1A3	188.4 ± 4.0	81.8 ± 6.9
1C1	ND	ND
1E1	0.27 ± 0.08	0.22 ± 0.03
2A1	12.7 ± 1.3	2.4 ± 0.1
2B1a	6.7 ± 1.5	3.3 ± 0.4
2B1b	3.3 ± 0.5	2.5 ± 0.1

ND = no detectable activity

Estrogen-Induced Carcinogenesis

K_m Values for 4-OH E1 (E2)

SULT Isoform	Apparent K_m Values ($\mu\text{M} \pm \text{S.E.}$)	
	4-OH E1	4-OH E2
1A1*1	53.8 ± 2.1	44.0 ± 5.2
1A1*2	53.3 ± 9.3	23.4 ± 3.7
1A1*3	40.4 ± 4.4	6.3 ± 0.1
1A2*1	17.1 ± 2.0	27.9 ± 0.7
1A2*2	65.1 ± 9.4	42.4 ± 2.7
1A2*3	5.1 ± 0.8	7.6 ± 2.0
1A3	32.4 ± 3.7	475.8 ± 93.8
1C1	ND	ND
1E1	0.31 ± 0.13	0.18 ± 0.06
2A1	20.4 ± 1.0	41.5 ± 19.5
2B1a	20.7 ± 1.8	45.2 ± 13.0
2B1b	13.6 ± 0.4	17.5 ± 1.0

ND = no detectable activity

Estrogen-Induced Carcinogenesis **Km Values for E1 and E2**

SULT Isoform	Apparent Km Values $\mu\text{M} \pm \text{S.E.}$	
	E1 (ESTRONE)	E2 (ESTRADIOL)
1A1*1	ND	31.3 ± 6.2
1A1*2	ND	84.6 ± 24.3
1A1*3	ND	21.1 ± 2.9
1A2*1	ND	28.6 ± 3.8
1A2*2	ND	145.4 ± 21.3
1A2*3	ND	18.3 ± 2.2
1A3	ND	ND
1C1	ND	ND
1E1	0.11 ± 0.1	0.097 ± 0.06
2A1	11.2 ± 0.9	12.4 ± 0.9
2B1a	ND	84.3 ± 7.6
2B1b	ND	60.9 ± 10.0

ND - Little or no detectable activity .

SULT1E1 Substrate Kinetics

<u>Substrate</u>	<u>K_m</u> (μ M)	<u>V_{max}</u> (Units/mg protein)	<u>V_{max}/K_m</u>
E2	0.097	6.88	70.9
E1	0.11	4.13	37.5
4-OH E2	0.18	6.04	33.6
2-OH E2	0.22	4.77	21.7
4-OH E1	0.31	2.35	7.6
2-OH E1	0.27	1.61	6.0



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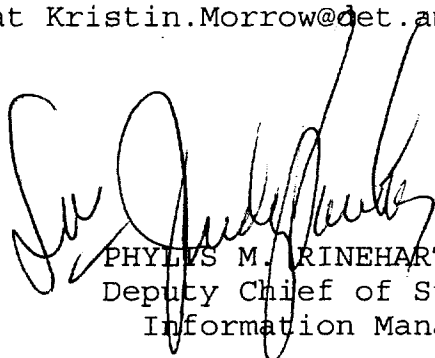
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